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Two principal lines of endeavor were pursued during the period covered by this report.

a. The search for small microorganisms which would be missed by the standard assay methods. Some work has been done on the characterization of the organisms found.

b. The characterization of Pleuropneumonia-like organisms as examples of very simple cells (with a possible relationship to primitive cells). Much of this work has centered on nutritional requirements in attempts to obtain growth media upon which the cells metabolic activities can be more thoroughly studied.

Our previous studies (1) indicated the utility of pourous cellulose ester membranes in the primary isolation of small cells. It was decided to use this technique as a first screening procedure. The chief reason for this is that terrestrial environments are so heavily contaminated with ordinary bacteria that some method is needed to eliminate them in looking for small forms. Material was collected from the following sources:

1. Estuarine waters
2. Marine waters
3. Pond waters
4. Arable soils
5. Nasopharyngeal washings from individuals with upper respiratory diseases
6. Plant homogenates.

The following three growth media were most frequently used in these studies.

1. Sea water medium containing 0.5% peptone, 0.1% yeast extract, and a complex mixture or chelated ions. This medium supported luxuriant growth of a very large proportion of the cultures isolated.

2. Sea water infusion of fresh minced beef heart, with the addition of 2% tryptose, adjusted to pH 8. This medium was sterilized by filtration through asbestos (Seitz) and dispensed aseptically as needed. This medium supports luxuriant growth of more cultures than any other, but some grew somewhat better in the sea water medium mentioned above.

3. A distilled water medium composed of 2% tryptose, 0.5% NaCl adjusted to pH 8. This medium after autoclave sterilization was supplemented with 0.5% glucose and 1% of a PPLO serum fraction. This medium supported the growth of only a few cultures, but those which grew in it usually grew feebly if at all in the other media.

As a preliminary control, 50 common strains of bacteria, which would grow on the sea water peptone, were subject to filtration (see next section for filtration procedure) and no filterable forms of these cultures were found. This also indicates that the techniques used were relatively free of contamination problems.

The general procedures used for filtering were as follows. Samples (if the initial samples were not liquid they were suspended in broth medium or saline solution) were serially filtered through Millipore filters of .45μ, .3μ and .22μ pore diameter. Filtration was carried out with a sterile Luer-Lok syringe fitted with a Swinney adapter. The filtrates were collected steriley and innoculated either into the various nutrient broths or on agar plates with the same nutrients.

Estuarine and marine waters almost always yielded filterable forms. Twenty of these have been isolated which continue to grow and give rise to filterable forms on the sea water beef heart growth medium. One estuarine strain grows only in tryptose. A number of other strains fail to yield filterable forms after culture in our media. All twenty strains are extremely pleomorphic under optical microscopy. Extremely unsatisfactory results have been obtained with electron microscopy which suggests that the cells are extremely fragile and do not withstand our fixing and drying procedures. However, only a very limited amount of work has been done in this area.

Our samples from fresh water ponds consistently failed to give filterable forms that could be subcultured. Growth was occasionally obtained in the original filtrates.

One filterable strain was obtained from soil and one from a plant homogenate.

Three nasopharyngeal samples yielded no filterable forms on the tryptose medium or on fresh beef heart infusion.

At present, the filterable strains are being subject to investigation to assess their relationship to the eubacteria (including L forms) and the pleuropneumonia-like organisms.

One important practical point arises in these studies. If one is preparing media to look for living forms in diverse environments, it is not sufficient to filter the medium even through 0.22μ pore size filters. Since there are forms in nature which pass these filters, there is always the danger of introducing them with the medium.

Secondly, it appears that in estuarine and marine waters there exists a submicroscopic class of living cells which will be missed by normal culture procedures. The failure to find such forms in other environments does not guaruntee their absence, as the growth medium used may be inadequate to insure their growth.

The problem of growth medium for pleuropneumonia-like organisms (PPLO) is illustrative of similar problems of a wide range of organisms. We have developed a defined medium for Mycoplasma laidlawii B which contains over 50 ingredients. Efforts are currently underway to simplify this medium. Some of the pertinent featurs we have studied are:

1. Amino acid toxicity. Considerable improvement in growth has been made by reducing the concentration of a number of amino acids.

2. Fatty acid toxicity. Although fatty acids are required (the present medium contains oleic, palmitic, myristic, linoleic and linolenic), growth is inhibited at relatively low concentrations of these nutrients, the order of 1 mg per liter. Some work has been done in purifying the fatty acids on thin layer chromatography and the results suggest that much of the toxicity may be due to impurities in the fatty acid preparations.

3. Polypeptide requirements. The medium used requires a peptide mixture which may be satisfied from a number of different sources. The simplest mixture used consists of two peptides of trypsinized ribonuclease (Lys-glu-thr-ala-ala-ala-lys and Thre-Thre glu NH_2-ala asp NH_2 lys).

Reference

1. Morowitz, H. J., Tourtellotte, M. E. and Pollack, M. E. J. Bact. 85, 134 (1963).